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Article

Latent Functions and Applications of Cytochrome P450 Monooxygenases from *Thamnidium elegans*: A Novel Biocatalyst for 14α-Hydroxylation of Testosterone

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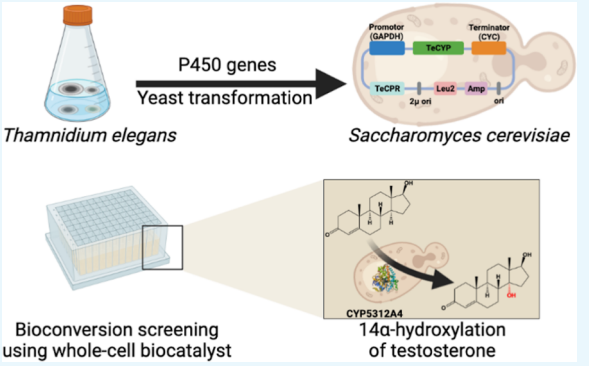
ABSTRACT: Cytochrome P450 monooxygenases (P450s) are ubiquitous enzymes with high availability and diversity in nature. Fungi provide a diverse and complex array of P450s, and these enzymes play essential roles in various secondary metabolic processes. Besides the physiological impacts of P450s on fungal life, their versatile functions are attractive for use in advanced applications of the biotechnology sector. Herein, we report gene identification and functional characterization of P450s from the zygomycetous fungus *Thamnidium elegans* (TeCYPs). We identified 48 TeCYP genes, including two putative pseudogenes, from the whole-genome sequence of *T. elegans*. Furthermore, we constructed a functional library of TeCYPs and heterologously expressed 46 TeCYPs in *Saccharomyces cerevisiae*. Recombinants of *S. cerevisiae* were then used as whole-cell biocatalysts for bioconversion of various compounds. Catalytic potentials of various TeCYPs were demonstrated through a functionomic survey to convert a series of compounds, including steroidal substrates. Notably, CYP5312A4 was found to be highly active against testosterone. Based on nuclear magnetic resonance analysis, enzymatic conversion of testosterone to 14α-hydroxytestosterone by CYP5312A4 was demonstrated. This is the first report to identify a novel fungal P450 that catalyzes the 14α-hydroxylation of testosterone. In addition, we explored the latent potentials of TeCYPs using various substrates. This study provides a platform to further study the potential use of TeCYPs as catalysts in pharmaceutical and agricultural industries and biotechnology.



INTRODUCTION

Cytochrome P450 monooxygenases (P450s) are a large superfamily of heme-containing monooxygenases that are distributed widely in living organisms ranging from bacteria to humans[1−3] and play numerous roles in secondary metabolism such as natural product biosynthesis,[4−6] steroidogenesis,[6−8] and detoxification of xenobiotics.[9−11] P450s are probably the most versatile biocatalysts in nature because of their wide variety of substrates and the types of reactions they catalyze.[3,12,13] In addition to their biological impact, P450s have attracted significant attention in the biotechnology sector as potential biocatalysts because P450s (i) contribute to the biosynthesis of natural compounds that have pharmaceutical and agricultural relevance;[14,15] (ii) catalyze site-specific conversion that chemical agents cannot achieve;[16−18] and (iii) play vital roles in the conversion or degradation of environmental pollutants by microbes.[19] Thus, functional studies on P450s should pave the way for advanced biology and biotechnology.

Steroids are essential substances that play important roles in physiological activities and are natural/potential substrates of P450s.[7,20,21] Steroidal drugs are used widely in treating people because of their medicinal properties, including antitumor, anti-inflammatory, antidiabetic, antimicrobial, and anticonvulsant properties.[22] The properties of steroids are differentiated based on the side chain at C17 and the level of oxygenation of the four rings of the steroidal core.[4] In addition, the hydroxyl groups in steroids affect their physiological functions. For example, the 7α-hydroxyl group is essential for regulating the immune system function and glucocorticoid action,[23] and the hydroxyl group at C14 confers steroids with carcinolytic and antigonadotropic activities.[24] Therefore, developing synthetic strategies for producing a wide variety of steroidal compounds is of significant interest. Microbial biotransformation can provide a powerful tool to achieve site-/regioselective modification of steroids; for example, several fungi such as *Cephalosporium aphidicola*,[25] *Aspergillus sydowii*,[26] and *Ulocladium chartarum*[22] have been shown to metabolize exogenous steroids to their hydroxylated derivatives.

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The fungal kingdom consists of a wide variety of eukaryotic microorganisms with an estimated size of 1.5 million species.[27] They inhabit a broad range of environments, and this has arisen from an evolutionary history, where fungi have developed secondary metabolic systems to survive in unique environments. Accordingly, fungal P450s exhibit astonishing diversity on the molecular and functional levels.[1,13,19,28−30] Among the four traditional fungal phyla, fungal species classified into Basidiomycota, Ascomycota, and Zygomycota generally encode a large number of P450 genes.[29] Many researchers have explored the function and potential utility of fungal P450s.[31−33] However, most studies on fungal P450s have focused on basidiomycetous and ascomycetous fungi. This focus can be attributed to specific species in such fungal phyla being extensively studied for potential applications, even from the pregenomic era. More recently, genome sequences of several fungi have been sequenced and released through the 1000 fungal genome project, providing a new research paradigm associated with the functionomics of enzymes. Under such circumstances, we explored the latent potentials of P450s from the zygomycetous fungus *Thamnidium elegans* (TeCYP), thus taking advantage of the genomic project. This fungus is found in typical habitats, such as stored meats, soil, and dung, and its whole-genome sequence is currently open to the public. Although some application studies such as fatty acid production have been reported using *T. elegans*, catalytic functions and the potential of TeCYPs have not been explored.[34−37]

Herein, we present functional information of TeCYPs that were uncovered by a genome-wide survey and functionomic investigation. We found 48 candidates, including two putative pseudogenes of TeCYPs, in the genomic database and isolated/generated 46 cDNAs encoding a mature open reading frame of P450. Using the full-length cDNA, we constructed expression plasmids of TeCYPs and developed a functional library in which the complete TeCYP (46 species) was heterologously expressed in *Saccharomyces cerevisiae* (*S. cerevisiae*). The yeast transformants harboring TeCYPs were used as biocatalysts for the conversion of various substrates. Notably, a comprehensive functional screen of the TeCYPs identified CYP5312A4, which converts testosterone to 14α-hydroxytestosterone. This is the first report of testosterone 14α-hydroxylation catalyzed by a fungal P450. Furthermore, this study addresses the molecular and functional diversity of TeCYPs, which should facilitate advanced biology and biotechnology of zygomycetous fungi.

RESULTS AND DISCUSSION

Identification and Isolation of CYP Genes from *T. elegans*. Possible coding sequences of TeCYPs in the *T. elegans* genomic database were searched for using the BLAST program. Based upon a bioinformatic survey, we found 48 possible TeCYP candidates that showed sequence similarity to known P450s (Table 1). However, two genes appeared to be pseudogenes because one (gm1.9774_g, Table 2) encodes a partial fragment of a P450, and the other (e_gw1.33.329.1, Table 2) has a possible mutation around an exon/intron boundary that disrupts the GT-AG rule. Thus, we focused on 46 candidates for further investigation (Table 2). According to the P450 nomenclature, the identified TeCYPs were classified into 23 families and 24 subfamilies.[38] Analysis of the phylogenetic relationship of TeCYPs with known P450s from basidiomycetous fungus *Postia placenta* and ascomycetous fungus *Aspergillus oryzae* revealed that TeCYPs represent a distinct cluster in the phylogenetic tree (Figure S1). Thus, *T. elegans* has increased the number of P450 superfamily members to meet its unique biology.

Table 1. Summary of TeCYPs in Numbers

description	number
gene candidates[a]	48
CYP Family	
family	23
subfamily	24
cDNA Amplification	
full-length cDNA	40
frame-shifted cDNA	1
not amplified	5
Synthesis of Engineered cDNA	
from cDNA	1
from gDNA	5
Heterologous Expression[b]	
confirmed by CO difference spectra	11
confirmed by bioconversion	19
total	30

[a]The numbers include two pseudogenes. [b]Six species were confirmed by both CO difference spectra and bioconversion.

We then aimed to isolate possible cDNAs encoding TeCYPs. Total RNA was obtained from the fungal mycelia grown in a synthetic liquid culture medium that stimulated transcription of a series of genes responsible for secondary metabolism.[39,40] Reverse transcription-polymerase chain reaction PCR (RT-PCR) was used successfully to amplify 40 cDNAs encoding a mature open reading frame of TeCYP. However, one candidate, CYP5206Q10, was amplified as an immature transcript whose open reading frame was shifted by a deleterious splicing event. In previous studies, we observed similar phenomena from basidiomycetous and ascomycetous genes.[41−43] The possible involvement of alternative splicing events in fungi was also suggested.[40] Thus, zygomycetous fungi appear also to possess sophisticated splicing mechanisms. Gene expression of five TeCYPs was not observed. These five TeCYPs are transcriptionally silent, at least in part, under the growth conditions used in this study. Therefore, cDNAs of frame-shifted and non-expressed candidates were rationally generated from genome DNA by removing intronic regions to encode a reliable P450 sequence. Namely, we were able to isolate/generate full-length cDNAs of all TeCYPs. More interestingly, we identified and isolated three isoforms of self-sufficient P450s from *T. elegans*, CYP5205A9, CYP5205A10, and CYP5205A11, which contain more than 1200 amino acids. Because self-sufficient P450s are distributed in the vast majority of fungal species,[44−46] these enzymes likely emerged at an early stage of fungal evolution. Furthermore, we isolated full-length cDNA encoding cytochrome P450 oxidoreductases (CPR) from *T. elegans* (TeCPR), which is the common redox partner of TeCYP. In general, fungi possess a small number of CPRs encoded by paralogous genes.[47,48] In this study, we found two CPR genes in the *T. elegans* genome and isolated these genes as full-length cDNAs (Supporting Information). One CPR (TeCPR_A) was used as a redox partner to construct a parent plasmid for the heterologous expression of TeCYPs in an *S. cerevisiae* strain.

Heterologous Expression of TeCYPs in *S. cerevisiae*. To functionally characterize TeCYPs in *S. cerevisiae*, we first

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Table 2. List of TeCYPs and TeCPRs

target[a]	protein ID[b]	scaffold/location[b]	length[c]	transcript[d]	accession number
CYP51F1	376054	2/2,017,883−2,019,769	515	FL	LC651335
CYP51F16	345413	21/87,862−89,559	414	FL	LC651327
CYP61A1	410878	3/1,456,682−1,458,971	515	FL	LC651339
CYP509G5	497853	32/346,292−348,594	522	FL	LC651355
CYP509H2	440800	29/311,357−313,314	535	FL	LC651346
CYP509H3	349524	33/258,653−260,555	531	FL	LC651359
CYP509H4	349584	33/241,180−243,191	530	FL	LC651329
CYP509H5	431146	33/234,789−236,828	534	FL	LC651362
CYP509H6	431208	33/299,789−301,900	529	FL	LC651344
CYP509R1	468007	32/343,812−346,031	507	NA	-
CYP509S1	339174	10/823,077−825,071	517	FL	LC651326
CYP5203A24	486532	7/594,744−596,518	505	FL	LC651350
CYP5203A25	373269	1/233,186−235,012	508	FL	LC651334
CYP5203A26	358519	6/206,257−208,182	509	FL	LC651330
CYP5203A27	439886	20/563,689−565,566	513	FL	LC651345
CYP5203B2	492882	9/168,226−170,134	509	FL	LC651352
CYP5203C1	416946	9/18,576−20,668	518	FL	LC651343
CYP5203C2	489815	2/930,401−932,431	511	FL	LC651351
CYP5203C3	360914	9/24,284−26,381	518	FL	LC651331
CYP5204A3	497980	33/289,609−291,349	511	FL	LC651356
CYP5205A9	428883	27/197,939−201,765	1211	FL	LC651365
CYP5205A10	383951	13/302,347−306,126	1200	NA	-
CYP5205A11	452797	13/448,411−452,310	1217	NA	-
CYP5206AA1	392775	40/23,330−25,263	509	FL	LC651337
CYP5206Q4	494294	13/704,515−706,433	524	FL	LC651353
CYP5206Q5	411225	4/242,717−244,835	529	FL	LC651341
CYP5206Q6	449517	4/253,590−255,558	529	FL	LC651347
CYP5206Q7	411232	4/245,516−247,595	527	FL	LC651340
CYP5206Q8	348619	30/328,886−330,730	501	FL	LC651328
CYP5206Q9	390667	30/325,998−327,870	502	FL	LC651336
CYP5206Q10	490844	4/258,183−250,102	503	FS	-
CYP5206Q11	449516	4/251,207−253,073	529	FL	LC651364
CYP5206Q12	395499	3/804,071−805,931	529	FL	LC651361
CYP5206X1	448619	2/1,281,377−1,283,282	524	FL	LC651363
CYP5206Y1	394862	2/1,276,837−1,278,711	508	FL	LC651360
CYP5206Z1	368023	23/366,404−368,390	521	FL	LC651333
CYP5207D1	365799	18/246,790−248,712	528	FL	LC651332
CYP5208A4	338189	9/1,090,714−1,092,413	507	FL	LC651357
CYP5209C2	406769	1/1,802,041−1,803,708	531	FL	LC651338
CYP5210A8	447479	1/64,413−66,339	542	FL	LC651358
CYP5211A4	435737	1/1,014,054−1,015,850	521	NA	-
CYP5212A3	437596	7/216,499−218,489	495	NA	-
CYP5312A4	497073	27/162,744−164,499	534	FL	LC651354
CYP5312A5	456647	36/41,853−43,633	533	FL	LC651349
CYP5312A6	455697	28/349,426−351,487	520	FL	LC651348
CYP5313A2	411472	4/554,778−557,042	519	FL	LC651342
e_w1.33.329.1	349823	33/237,344−239,362	518	ND	-
gm1.9774_g	467209	28/14,784−15,755	78	ND	-
TeCPR_A	369825	29/77,584−80,266	714	FL	LC656311
TeCPR_B	438695	12/262571−264814	712	FL	LC656312

[a]The CYP name was assigned by the P450 nomenclature committee. The two pseudogenes are listed with gene numbers (e_w1.33.329.1 and gm1.9774_g) assigned in the database. [b]Protein ID, scaffold, and location are matched with the database v1.0. (https://mycocosm.jgi.doe.gov/Thaele1/Thaele1.home.html). [c]The number indicates amino acid length. [d]FL, full-length; FS, frame-shifted; NA, not amplified; and ND, not done.

constructed a parental plasmid pLYGII-TeCPR that was derived from commercially obtained pYES2 and then each TeCYP was incorporated into pLYGII-TeCPR (see the details in the Experimental Section and the Supporting Information). Briefly, pLYGII-TeCPR was designed to allow simultaneous expression of TeCYPs and its native redox partner TeCPR. Using the cDNAs of TeCYPs, we obtained 46 distinct plasmids that were transformed into an *S. cerevisiae* strain for heterologous expression. The growth curves of the transformants were similar to that of the host strain (data not shown), indicating that TeCYPs exhibited no deleterious effect on yeast homeostasis. Carbon monoxide (CO) difference

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spectra of transformants were analyzed to evaluate the heterologous expression of TeCYPs. Here, the active form of P450 displays an absorption maximum at ∼450 nm, which is attributed to the proximal thiolate ligand coordinating to the heme.[49,50] Spectral analysis revealed that at least 11 TeCYPs were expressed abundantly in *S. cerevisiae* (Figure S2). Although typical CO difference spectra were not detected for the other 35 TeCYPs, these TeCYPs were still expected to be expressed at low levels. This likely low-level yet undetected expression is supported by the experimental observation that endogenous CYP51, a housekeeping gene, was constitutively expressed in the host cells but not at diagnostic levels (data not shown). As described below, several TeCYPs converted potential substrates even though CO difference spectra did not confirm their expression. For example, 13 TeCYPs showed catalytic activities toward tentative substrates even though their CO difference spectra were not detected. Combining the data from spectral analysis and bioconversion reaction activity, we concluded that at least 52.2% (24 out of 46) TeCYPs were active in *S. cerevisiae*.

Bioconversion Reaction of Various Compounds by TeCYPs in *S. cerevisiae*. For high-throughput screening, we prepared a functional library of using 46 TeCYPs in which each transformant harboring an expression plasmid was grown and compartmentalized using a square-shaped 96-well plate.[41,51] The functional library was replicated and used for reaction screening, where the transformants were incubated with a wide variety of compounds, and the resultant metabolic products were analyzed by high-performance liquid chromatography (HPLC). In this study, we found enzymatic activities of different TeCYPs against 13 tentative substrates recruited from different chemical categories such as steroids, pharmaceuticals, plant-related compounds, and polycyclic aromatic hydrocarbons (PAHs). The catalytic potentials of the TeCYPs revealed in this study are summarized in Table 3.

Table 3. Summary of Catalytic Potentials of the TeCYPs against Various Compounds[a]

substrate	TeCYPs (substrate conversion, %)
	Steroids
testosterone	CYP509G5 (2), CYP509H2 (10), CYP5312A4 (95), CYP5312A5 (16)
progesterone	CYP509G5 (5), CYP509H2 (3), CYP5312A4 (95), CYP5312A5 (11)
ADD	CYP509G5 (6), CYP509H2 (14), CYP5312A4 (95), CYP5312A5 (11)
methyltestosterone	CYP509H2 (4), CYP5312A4 (95), CYP5312A5 (14)
	Plant-Related Compounds
abietic acid	CYP5204A3 (3), CYP5206Q5 (5), CYP5206Z1 (3), CYP5312A5 (3)
dehydroabietic acid	CYP509H2 (44), CYP5312A5 (38)
7-ethoxycoumarine	CYP509G5 (2), CYP5206Q8 (2), CYP5206Q11 (3), CYP5312A4 (4)
ferruginol	CYP509H2 (10), CYP509H4 (5), CYP5207D1 (9)
	Pharmaceutical Compounds
diclofenac	CYP509H2 (5)
ibuprofen	CYP509H2 (95), CYP509H3 (40), CYP5205A10 (95), CYP5205A11 (95), CYP5206Q4 (75), CYP5206Q5 (75), CYP5206AA1 (80)
	Polycyclic Aromatic Hydrocarbons (PAHs)
dibenzothiophene	CYP509H2 (2), CYP509H4 (3), CYP5206Q4 (3), CYP5206Q6 (3), CYP5206Q8 (3), CYP5206Q11 (4), CYP5206Q12 (5), CYP5206Z1 (4)
phenanthrene	CYP5206Q6 (3), CYP5206Q8 (5), CYP5206Q10 (5), CYP5206Q11 (5), CYP5206Z1 (1)
pyrene	CYP509H2 (17), CYP5206Q11 (28)

[a]The substrate conversion percentage was calculated based on peak intensities of representing residual substrates in the samples, and the values are presented in the parentheses. The bioconversion reactions were conducted with a substrate concentration of 0.5 mM in 750 μL of medium using a 96-square-shaped deep-well plate. Ibuprofen conversion was performed with a concentration of 0.125 mM because of its toxicity for yeast.

A functionomic survey showed that several TeCYPs exhibit significant activities against steroidal substrates. As shown in Figure 1, CYP5312A4 displayed superior activity in converting testosterone. Similarly, progesterone and 1,4-androstadiene-3,17-dione (ADD) were potential substrates of CYP5312A4 (Figure S3). Interestingly, the CYP5312 family from *T. elegans* and of the CYP512 family found in basidiomycetous fungi showed a phylogenetic relationship (Figure S1). Several basidiomycetous P450s belonging to CYP512 have exhibited catalytic activities toward steroidal compounds.[41,53] Furthermore, both CYP5312 and CYP512 families are phylogenetically close to lanosterol 14α-demethylase (CYP51), which is a housekeeping gene involved in ergosterol biosynthesis by eukaryotic fungi. Thus, in nature, CYP5312 may play biological roles in fungal steroid and/or triterpenoid metabolism. Functional screening revealed that CYP509H2, CYP5312A5, and CYP509G5 also showed catalytic activities against testosterone, progesterone, and ADD, giving several products even though the conversions proceeded at low levels when compared with that of CYP5312A4. Thus, we cannot scale up the bioconversion reaction of CYP509H2, CYP5312A5, and CYP509G5 to a large volume for further structural analysis of hydroxylated testosterone (Figure S4). Furthermore, CYP5312A4, CYP5312A5, and CYP509H2 catalyzed the hydroxylation of 17α-methyltestosterone (Figure S5). Although CYP5312A4, CYP509H2, CYP5312A5, and CYP509G5 yielded different product profiles during steroid conversions, these TeCYPs recognized structural features shared with testosterone, progesterone, and ADD (Scheme 1). In contrast, these four TeCYPs did not convert dehydroepiandrosterone (DHEA) despite its structural similarity to testosterone, progesterone, and ADD. Although further investigations are required to elucidate reaction mechanisms, the presence of the carbonyl group at the C3 position in steroidal compounds appears to be essential for substrate recognition by the four TeCYPs (Scheme 1). In previous studies, we have also demonstrated testosterone conversion using P450s from basidiomycetous and ascomycetous fungi.[41,52] Combining these findings indicates that P450s catalyzing steroid conversions are distributed widely in the fungi kingdom.

The chemical structures of hydroxylated products of testosterone were analyzed by performing a bioconversion reaction of testosterone in 1000 mL culture (ten 100 mL cultures in 500 mL baffled flask). Under these conditions, approximately 10 mg of the major product was successfully purified. We then conducted an nuclear magnetic resonance (NMR) analysis of the purified compound produced by CYP5312A4. Based on this NMR analysis, we identified the product as 14α-hydroxytestosterone. As shown in Figure 2, the ^{13}C NMR spectrum of the product was identical to literature data (Table S1),[22,25,26] and ^{1}H NMR, distortionless enhancement by polarization transfer, ^{13}C NMR, and two-dimensional NMR spectra (Figures S6−S10) consistently agreed with the production of 14α-hydroxytestosterone by CYP5312A4.

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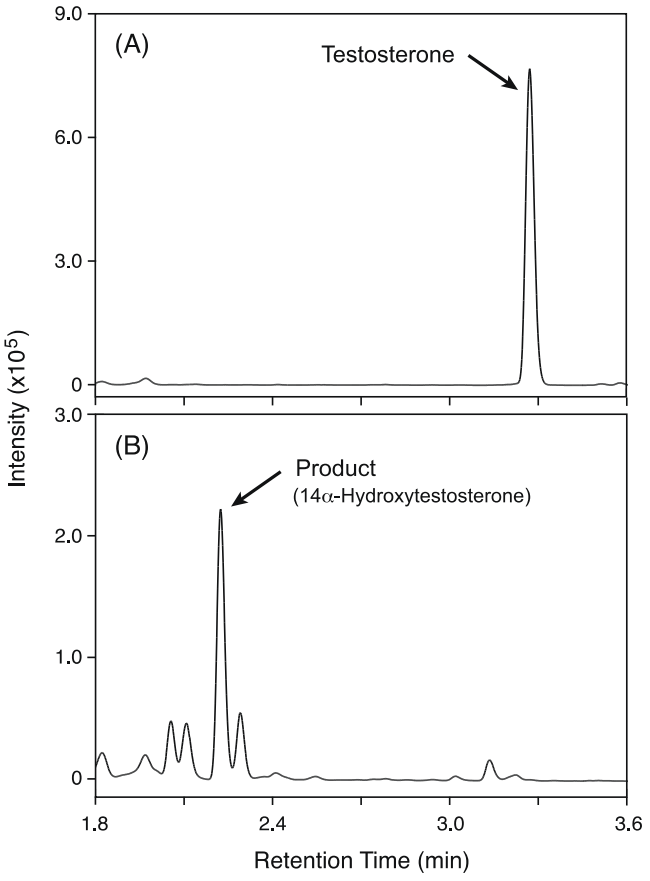

Figure 1. HPLC analysis of the bioconversion reaction of testosterone catalyzed by *S. cerevisiae* whole-cell catalyst expressing CYP5312A4. (A) *S. cerevisiae* harboring pLYGII-TeCPR without TeCYP was used as the control. The arrow indicated the peak of testosterone. (B) *S. cerevisiae* harboring pLYGII-TeCPR-CYP5312A4. The arrow indicated the major product formed from CYP5312A4-catalyzed 14α-hydroxylation of testosterone.

Although it is possible that several structural isomers of hydroxytestosterone (molecular weight: 304) concomitantly appeared as minor products (Figure S11), CYP5312A4 showed high specificity in converting testosterone to the 14α-hydroxylated derivative. Interestingly, CYP5312A4 preferred to catalyze 14α-hydroxylation of testosterone even though the tertiary C14 atom is sterically demanding (Figure S12). Thus, substrate recognition by CYP5312A4 must be highly tuned to accommodate testosterone with a unique orientation. Remarkably, 2α-, 2β-, 6α-, 6β-, 7α-, 7β-, 11α-, 11β-, 12β-, and/or 15α-hydroxylation of steroids has been reported for fungal P450s.[17,53−55] Moreover, as far as we understand, bacterial and human P450s also reported to be capable of catalyzing the hydroxylation of testosterone at 2α-, 2β-, 6β-, 7β-, 11β-, 12β-, 15β-, and 16α- and 17-hydroxylation to form 17-ketoproduct.[4,56−59] Two previous studies have reported the steroid 14α-hydroxylation activity of the P-450lun from *Cochliobolus lunatus* toward steroidal compounds cortexolone and androstenedione.[60,61] However, no reports have described the 14α-hydroxylation of testosterone catalyzed by P450. Therefore, this is the first demonstration of P450-mediated 14α-hydroxylation of testosterone. The functional identification of CYP5312A4 complements earlier notions that several fungi can convert testosterone to 14α-hydroxytestosterone even though metabolic systems involved in the reaction have not been identified.[22,25,26] Thus, functionomic studies may highlight latent potentials of fungal P450s hidden behind fungal biology.

Besides studying steroid conversion, functional screening also revealed interesting insights into the catalytic potentials of TeCYPs (Table 3; see also Figure S13). For example, TeCYPs belonging to the CYP5206 family converted PAHs such as phenanthrene, dibenzothiophene, and pyrene. Thus, these TeCYPs are potential tools for biotechnology applications such as environmental remediation. Furthermore, CYP509H2 showed catalytic activity over a broad range of substrates such as plant-derived diterpenoids (dehydroabietic acid and ferruginol), pharmaceuticals (diclofenac and ibuprofen), PAH (pyrene), and steroids (testosterone, progesterone, and ADD) despite poor correlation in their chemical structures. Based on the phylogenetic analysis (Figure S1), the CYP509 family found in *T. elegans* and CYP5150 diversified in basidiomycetous fungi have likely emerged and evolved from a common ancestral P450 gene. Interestingly, several basidiomycetous P450s belonging to the CYP5150 family exhibit catalytic activities toward a broad range of substrates.[41,53] Although further investigations are required to obtain a comprehensive understanding of sequence/structure/activity relationships of P450, these findings suggest that the versatile function of CYP509H2 is associated with the environmental adaptation of *T. elegans*. Thus, further investigations using the functional library of TeCYPs should advance our understanding of the biology of *T. elegans* and potential biotechnology applications.

CONCLUSIONS

We identified and isolated genes encoding P450s from *T. elegans* (TeCYPs) and demonstrated their heterologous expression in *S. cerevisiae*. We isolated/obtained full-length cDNAs of 46 TeCYPs, indicating that the whole P450 in *T. elegans* was successfully cloned. Furthermore, TeCYPs were heterologously expressed in *S. cerevisiae*, facilitating the rapid and comprehensive screening of TeCYP functions. As indicated by the CO-difference spectra and bioconversion screening analysis, at least 52.2% of TeCYPs were functionally expressed in *S. cerevisiae*. The bioconversion screening reaction of substrates was performed using various compounds to confirm the reactivity of the CYPs against different types of compounds. The functionomic study highlighted that CYP5312A4 showed significant activity against testosterone, progesterone, and ADD. Interestingly, CYP5312A4 catalyzed 14α-hydroxylation of testosterone, which may be a rare and unique reaction in nature. This is the first time a fungal P450 has been identified to catalyze 14α-hydroxylation of testosterone. This study advances the potential use of fungal P450s as catalysts in pharmaceutical and agricultural industries and biotechnology.

EXPERIMENTAL SECTION

Chemicals. Abietic acid, ADD, DHEA, methyltestosterone, phenanthrene, and progesterone were purchased from Tokyo Chemical Industry (Tokyo, Japan). Dehydroabietic acid, dibenzothiophene, 7-ethoxycoumarin, ibuprofen, and testosterone were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Pyrene was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Diclofenac was purchased from Combi-Blocks USA (San Diego, CA, USA). Ferruginol was kindly provided by Dr. H. Suhara (Miyazaki Prefectural Wood

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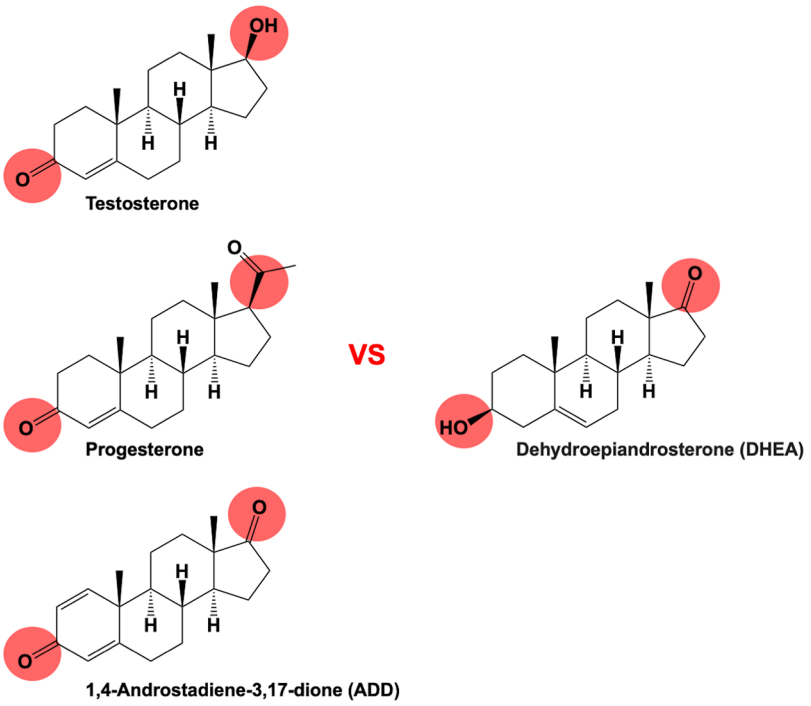
Scheme 1. Chemical Structure of Testosterone, Progesterone, ADD, and DHEA[a]



[a]Functional groups in the compounds that may affect the activity of TeCYPs are highlighted in red.

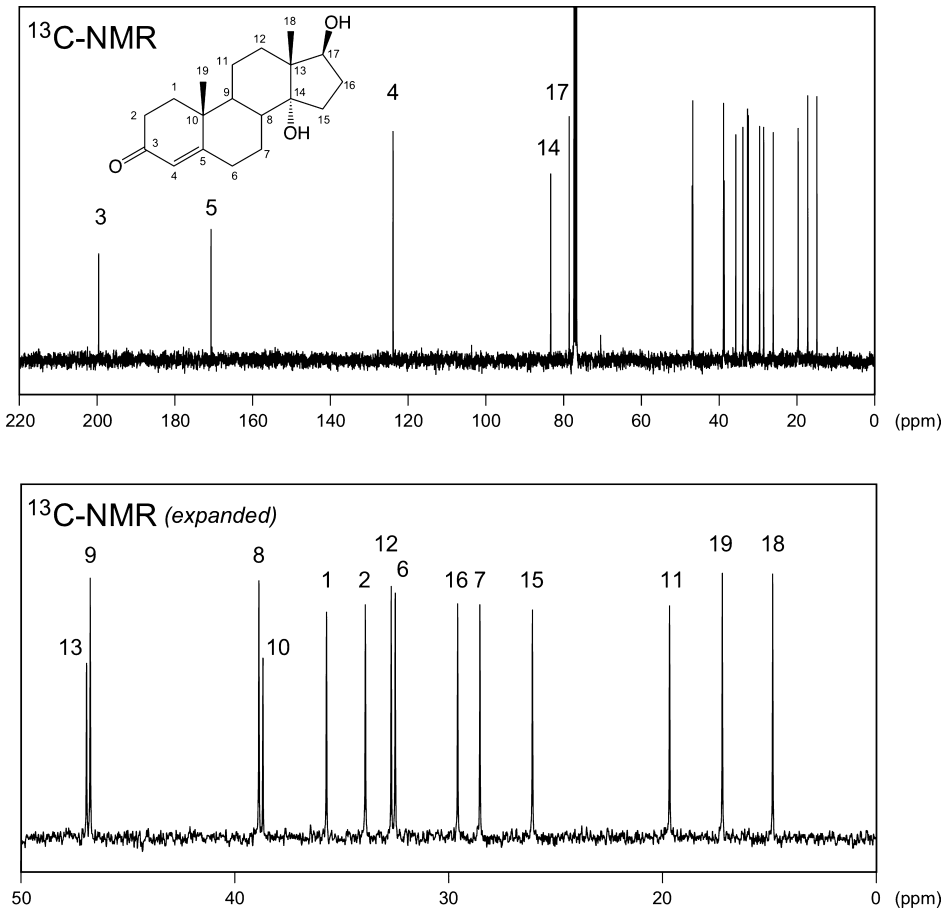


Figure 2. ^{13}C NMR spectra of 14α-hydroxytestosterone produced by CYP5312A4.

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Utilization Research Center, Japan). Yeast nitrogen base without amino acids was purchased from Formedium (Hunstanton, UK). Dropout supplements (DOS) were purchased from TaKaRa Bio USA (Mountain View, CA, USA). Custom-synthesized oligonucleotide primers were obtained from Sigma-Aldrich Japan. All other chemicals were of reagent grade. Deionized water was obtained using a Barnstead Smart2Pure System (Thermo Fisher Scientific, Waltham, MA, USA).

Gene Identification of TeCYPs. Possible coding sequences of TeCYPs were found in the US Department of Energy Joint Genome Initiative database based on sequence similarity to known P450s (https://mycocosm.jgi.doe.gov/Thaele1/Thaele1.home.html). To evaluate annotation accuracy, we identified the P450s' signature sequence (F−x−x−G−x−x−x−C−x−G) in the heme-binding domain, the E−x−x−R motif in the K-helix, a conserved Thr in the center of the I-helix, and the hydrophobic transmembrane domain in the N-terminal region. The sequences for the transmembrane domain were analyzed using SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/)[62] and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/).[63] Gene candidates that lacked sequences corresponding to these conserved regions were judged by their overall sequence similarity to known P450s.

Amplification of cDNA Encoding TeCYP and TeCPR. *T. elegans* (ATCC 42612) was grown from hyphal inoculum at 25 °C in a stationary culture (10 mL medium in a 100 mL Erlenmeyer flask) under aerobic conditions. Kirk medium (1% [w/v] glucose and either 1.2 or 12 mM ammonium tartrate as carbon and nitrogen sources, respectively, pH 6.0) was used in this study based on previous reports.[39−41] Total RNA was extracted individually from 4, 6, and 8 day old mycelia using the acid guanidium−phenol−chloroform method[64] and further purified using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The RNA concentration was calculated from the absorbance at 260 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Purified RNA was then applied for RT using ReverTra Ace (TOYOBO, Osaka, Japan) in the presence of oligo(dT) primers (5′-TTTTTTTTTTTTTTTTTTV-3′; V = A, C or G). The RT reaction was carried out according to the manufacturer's protocols. The reaction mixtures were stored at −20 °C until PCR amplification. The following amplification of the target gene was performed by nested PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs Japan, Tokyo, Japan) and/or KOD FX Neo (TOYOBO), according to the manufacturer's protocols. The reaction conditions for PCR are detailed in the Supporting Information. The primer sequences used for RT-PCR are listed in Table S2. Experimental strategies for cDNA cloning are illustrated in Figure S14. After PCR amplification, gene fragments were cloned into the EcoRV/SmaI site of pBluescript II KS(−) and sequenced using an automated DNA Sequencer (ABI 3730xl DNA Analyzer; Applied Biosystems, Waltham, MA, USA). The amino acid and nucleotide sequences of isolated TeCYPs and TeCPR were deposited in the DNA Data Bank of Japan (Table 2, see also Supporting Information). Experimental protocols for the preparation of synthetic cDNA for CYP509R1, CYP5205A10, CYP5205A11, CYP5206Q10, CYP5211A4, and CYP5211A5 are also provided in the Supporting Information (Figure S15, the list of primers is also available in Table S3).

Heterologous Expression of TeCYPs. For heterologous expression of TeCYP in *S. cerevisiae*, a parental plasmid pLYGII-TeCPR was generated from commercially obtained pYES2 (Thermo Fisher Scientific). Experimental procedures for preparing pLYGII-TeCPR are detailed in the Supporting Information (the list of primers is also available in Table S4). Briefly, pLYGII-TeCPR containing *Leu2* for auxotrophic selection, the glyceraldehyde-3-phosphate dehydrogenase promoter (derived from *Zygosaccharomyces rouxii*), and the cytochrome *c* 1 terminator for expression of the TeCYP and TeCPR genes was linked with the promoter/terminator of alcohol dehydrogenase 1. The coding sequence of each TeCYP was reamplified by PCR from the cloning vector. Nucleotide sequences of the primers used for TeCYPs from pBluescript II KS (−) plasmids are available in the Supporting Information (Table S5). The amplified cDNAs were then ligated into the yeast expression vector pLYGII-TeCPR linearized with PshAI/SpeI using the In-Fusion HD cloning kit (TaKaRa Bio USA). Figure S16 illustrates the construction of the TeCYP expression plasmids. Transformation of the expression plasmids into *S. cerevisiae* InvSc1 was conducted using a modified lithium acetate method, as described in the Supporting Information. Positive transformants were isolated by auxotrophic selection using synthetic dextrose agar plates. A fresh transformant was inoculated into 0.75 mL synthetic dextrose liquid (SDL) medium consisting of 8% (w/v) glucose, 2.68% (w/v) yeast nitrogen base without amino acids, and 0.1% (w/v) DOS without leucine (Leu) in a square-shaped 96-well plate, and these cultures were grown simultaneously for 3 days in a Micro Bio Shaker (TAITEC, Koshigaya, Japan) at 28 °C. After incubation, each culture was mixed with 0.75 mL potassium phosphate (10 mM, pH 7.0) containing 40% glycerol and stored at −80 °C. The CO difference spectra of the transformants were recorded on a UV−Vis spectrophotometer equipped with a head-on photomultiplier (U3900H, Hitachi, Tokyo, Japan).

Bioconversion Screening of CYPs against Various Substrates. The bioconversion of substrates was initiated by inoculating a 20 μL yeast culture into 0.75 mL of SDL medium containing a substrate (0.5 mM) in a square-shaped 96-well plate and incubating this culture in a Micro Bio Shaker at 28 °C and 1,300 rpm for 3 days. The bioconversion reactions were terminated by adding acetonitrile (0.75 mL) to the culture. The yeast cells were removed by centrifugation (1,300*g*) and filtration (0.45 μm), and the bioconversion products were analyzed by HPLC.

Large-Scale Production and Purification of 14α-Hydroxytestosterone. A large-scale bioconversion was conducted in 100 mL of medium in a 500 mL baffled flask. Ten baffled flasks were used to prepare 1 L of culture medium. Then, 400 μL of yeast (expressing CYP5312A4) culture was seeded into 100 mL SDL medium containing 0.5 mM testosterone and incubated in a shaking incubator (140 rpm) at 28 °C for 3 days. After incubation, the yeast cells were removed by centrifugation (10,000*g*) and the products were then extracted by ethyl acetate. After solvent evaporation, the crude extracts containing the target product were dissolved in hexane/ethyl acetate (1:1) and separated by flash chromatography (hexane/ethyl acetate gradient) using a FlashPure Select silica column (BUCHI, Flawil, Switzerland). The target product was finally purified by preparative HPLC (water/methanol gradient) using an Inertsil ODS-HL (GL Sciences, Tokyo, Japan). Chromatographic purification was carried out

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using a Pure C-850 FlashPrep system (BUCHI), and chromatographic profiles were monitored by evaporative light scattering and UV detection.

Instruments. HPLC analysis was carried out using a Prominence UFLC system (Shimadzu) consisting of two pumps (LC-20AD), an autoinjector (SIL-20AC HT), a UV detector (SPD-20A), and a column oven (CTO-20A). Chromatographic separation was performed using an Inertsil ODS-3 column (GL Sciences; 4 μm; 3.0 × 75 mm) with a column temperature of 40 °C. The mobile phases for HPLC were (A) water with 0.05% [v/v] phosphoric acid and (B) acetonitrile. The mobile phase gradient was as follows: 0−0.2 min, 10% B; 0.2−3.2 min, 10−40% B; 3.2−3.6 min, 40−100% B; 3.6−4.0 min, and 100% B. The flow rate was 1.5 mL/min. An ultraviolet (UV) monitor was used for detecting the products. NMR spectra were acquired with a JNM-ECZ400 (JEOL, Tokyo, Japan) and analyzed using Delta NMR software (JEOL). Chemical shifts were expressed as parts per million downfield from the internal standard tetramethylsilane. Samples were dissolved in deuterated chloroform.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00430.

Reaction conditions for nested PCR; preparation of synthetic cDNAs; construction of the pLYGII-TeCPR plasmid; transformation of yeast by a modified lithium acetate method; the culture medium used for auxotrophic selection of yeast transformants; literature data of ^{13}C NMR signals for 14α-hydroxytestosterone; list of primers used for cDNA isolation, generating synthetic cDNAs, construction of pLYGII-TeCPR, and constructing pLYGII-TeCPR-TeCYP expression plasmids; phylogenetic analysis of TeCYPs; CO difference spectra of *S. cerevisiae* expressing TeCYPs; catalytic activities of CYP5412A4 against progesterone and ADD; catalytic activities of CYP5312A5, CYP509H2, and CYP509H5 against steroidal substrates; catalytic activities of CYP5412A4, CYP5312A5, and CYP509H2 against 17α−methyltestosterone; ^{1}H NMR spectrum of 14α-hydroxytestosterone; DEPT ^{13}C NMR spectra of 14α-hydroxytestosterone; COSY spectrum of 14α-hydroxytestosterone; HMQC spectrum of 14α-hydroxytestosterone; HMBC spectrum of 14α-hydroxytestosterone; GC−MS analysis of metabolites produced by CYP5412A4; proposed reaction mechanism for 14α-hydroxylation of testosterone; bioconversion of various compounds catalyzed by TeCYPs; experimental strategies for cDNA cloning and generating synthetic cDNAs; schematic illustration for the construction of pLYGII-TeCPR; and cDNA and deduced amino acid sequences of TeCYPs and TeCPR (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ADD, 1,4-androstadiene-3,17-dione; CO, carbon monoxide; CPR, cytochrome P450 oxidoreductase; DOS, dropout supplements; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; P450, cytochrome P450 monooxygenase; PAH, polycyclic aromatic hydrocarbon; SDL, synthetic dextrose liquid; TeCYPs, *Thamnidium elegans* CYP P450s

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